A METHOD FOR THE DETECTION OF *d*-TUBOCURARINE, GALLAMINE, DECAMETHONIUM AND SUCCINYLCHOLINE IN BIOLOGICAL MATERIALS

MODIFICATION AND DEVELOPMENT

ANGELO FIORI
Istituto di Medicina Legale e delle Assicurazioni, Università Cattolica del Sacro Cuore, Via della Pineta Sacchetti 644, Roma (Italy)

MARIO MARIGO
Istituto di Medicina Legale e delle Assicurazioni, Università di Padova, via Falloppio 16, Padova (Italy)

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In a previous paper[1] a method was described for the detection of some synthetic curares, *e.g.* gallamine, decamethonium and succinylcholine, in biological materials based on the purification of the aqueous extracts by ion exchange separation on Amberlite IRC 50 columns and subsequent paper and thin-layer chromatographic identification. Biological tests on mice were then applied using the eluates from the chromatograms.

Some difficulties were met in the practical application of the method, especially when *d*-tubocurarine was present. The drawbacks are the following:

(1) The separation on Amberlite IRC 50 columns requires a large volume of 2 *N* HCl for a complete elution of the three synthetic curares; this stage and the subsequent evaporation are tedious and unpractical for routine work;

(2) The residue after evaporation of the eluates is often too rich in chlorides which interfere with the paper and thin-layer chromatography;

(3) *d*-Tubocurarine is strongly adsorbed on the cation exchanger so that only minimal amounts can be recovered on eluting with 2 *N* or 4 *N* HCl.

A simplified procedure was therefore adopted and some additional modifications made so that the method can now be regarded as satisfactory for forensic purposes.

MATERIALS AND METHOD

The following commercial products were used: Sincurarina Farmitalia (gallamine triiodide, 20 mg/ml), Syncurine Wellcome (decamethonium iodide, 2 mg/ml), Midarine Wellcome (succinylcholine chloride, 50 mg/ml) and Tubarine Wellcome (*d*-tubocurarine chloride, 10 mg/ml). The isolation procedure described below was developed for samples of urine, blood and human homogenized organs to which known amounts of the curares had previously been added. Choline and acetylcholine chlorides (Merck) were also used for paper and thin-layer chromatography.

Isolation

The sample to be treated by cation exchange purification is prepared in different ways according to the organ or body fluid to be examined.

Urine. This is filtered and employed directly for ion exchange separation of the curares. Previous extraction with ether both at acid and at basic pH, is advisable when organic solvent—soluble poisons are suspected to be present. The *d*-tubocurarine and the three synthetic curares still remain after the extractions in the aqueous layer, which is now used for the ion exchange separation.

Blood. Usually 50 ml is deproteinized by adding 1 volume of 20 % TCA, centrifuged and the supernatant filtered. The subsequent steps are the same as for urine.

Organs. Brain, liver, kidney, heart, muscle, lung are homogenized either separately or together. The homogenate (50–100 g) is deproteinized with 20 % aqueous TCA (v/v) and treated as for blood and urine.

When a complete research on viscera for non-volatile poisons is required, the *Stas-Otto process* is successfully employed according to the three group separation method (acids, ether soluble bases, chloroform soluble bases). Only minimal amounts of curares (especially *d*-tubocurarine) can be found in the chloroform extract, the major part remaining in the highly impure aqueous layer. In fact, synthetic curares belong to the water-soluble, solvent-insoluble group of poisons such as sulfanilamide, sulfodiazine, salicin, digitonin, etc., and *d*-tubocurarine is an amphoteric compound almost insoluble in ether and chloroform.

Cation exchange purification

The aqueous extracts obtained as described above are adjusted to pH 6, 1/3 volume of Amberlite IRC 50 (H) resin (analytical grade) is added and the suspension stirred 4 or 5 h in a beaker. The supernatant is discarded and the resin carefully washed three or four times with distilled water, 30 min each time.

The elution of *gallamine, succinylcholine and decamethonium* is effected by stirring the washed resin for 1 h with two volumes of 2 *N* HCl. Three additional elutions are made and the four fractions collected and filtered.

At this point almost all the *d-tubocurarine* is still adsorbed on the resin. It can easily be eluted by stirring three times, each time for 1 h, with successive fractions of 50 % alcoholic 2 *N* ammonia. The fractions are collected and filtered.

Precipitation of the curares as reineckates

The 2 *N* HCl eluates are usually too rich in chlorides so that the residues after evaporation to dryness cannot easily be chromatographed. The curares are therefore precipitated as reineckates according to the technique of KLEIN AND GORDON[6] by adding 1 ml of 20 % sulphuric acid and 3 ml of 2 % ammonium reineckate solution in water to 10 ml of the eluate. After standing for 1 h in an ice box, to allow for a complete precipitation of the pink reineckates, the tubes are centrifuged for 10 min, the supernatant is decanted and the precipitates are washed twice with water, each time centrifuging for 2 min, decanting and removing excess liquid with filter paper.

The eluates in 2 *N* ammonia can be evaporated directly or, alternatively, used for the Reinecke salt treatment.

Quantitative determination

Acetone is added to the washed and dried precipitate so that the volume

measures 10 ml after complete solution of the reineckate. Optical density is measured at 525 mμ as the Beer-Lambert law holds at this wavelength, from a concentration of 0.25 to 1.25 mg per ml (see also ZAIMIS[8]). Alternatively the LEE KUM-TATT equations[4] may be employed when the nature of the curare is already known.

Regeneration of the conjugate curares from their reineckates

A slight excess of a saturated aqueous solution of silver sulphate is added to the reddish-pink acetone solution and the precipitate of silver reineckate is removed by centrifugation and filtration. A 4% (w/v) barium chloride solution is added dropwise to the filtrate kept on a gently boiling water bath; barium sulphate and silver chloride are thus simultaneously precipitated. Any excess of barium chloride is to be avoided. The precipitate is again removed and the filtrate is evaporated to dryness; a few μl of distilled water are then added for the further chromatographic steps.

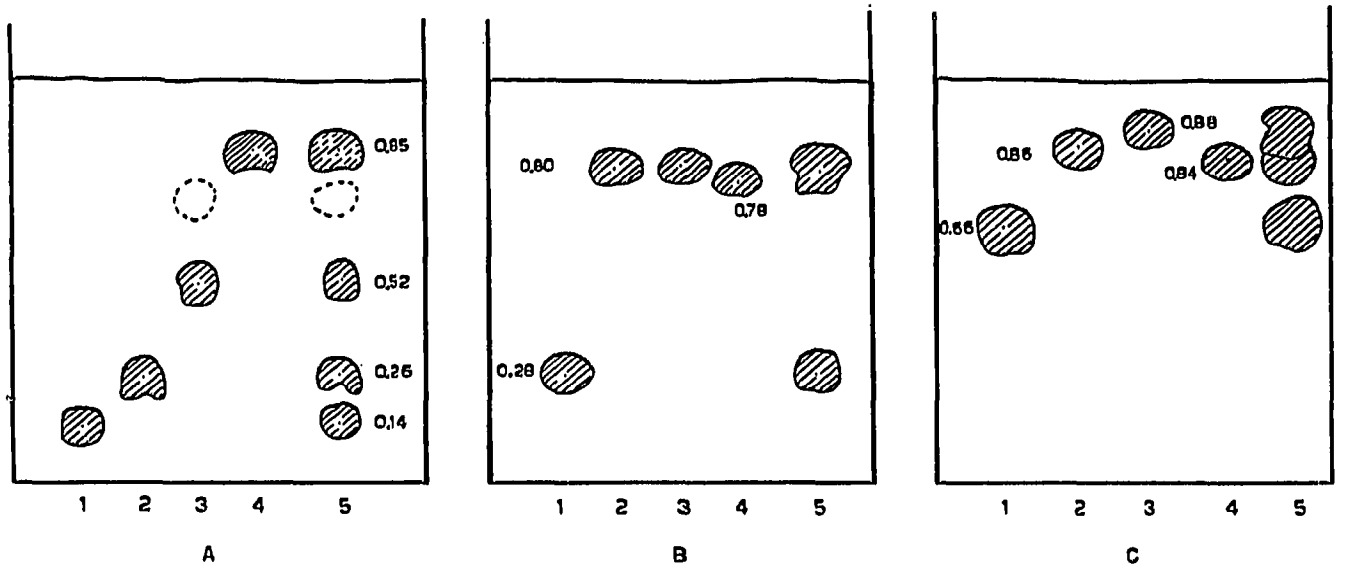

Fig. 1. Thin-layer chromatography of curare and some curare-like drugs. 1 = Succinylcholine; 2 = decamethonium; 3 = gallamine; 4 = *d*-tubocurarine; 5 = a mixture of 1–4. Detection: iodoplatinate. (A) adsorbent: alumina Woelm, acid; solvent: chloroform–methanol (80:20). (B) adsorbent: alumina Woelm, basic; solvent: methanol–chloroform (80:20). (C) adsorbent: Alumina G Merck; solvent: methanol–acetic acid–water (92:3:5).

Chromatographic identification

Paper chromatographic identification. This is carried out on Whatman No. 1 paper with *n*-butanol–acetic acid–water (40:10:50) as solvent. Iodoplatinate reagent is employed to detect the spots. The average R_F values for the commercial curare and curare-like drugs and related compounds are given in Table I.

Thin layer chromatography. This is performed on alumina layers. Silica gel layers do not permit the separation of the four curares as they do not migrate from the starting line with a great number of the usual solvents or, in the case of *d*-tubocurarine, it only migrates a few millimeters. On the contrary alumina (especially the cation exchanger "acid alumina") is a suitable adsorbent for TLC of the curare and curare-like drugs. 20 × 20 cm plates can be prepared by suspending 10 g of alumina in 25 ml

of ethyl alcohol–water (9:1). Fig. 1 indicates the separation obtained with alumina Woelm (acid and basic) and with Aluminium Oxide G Merck. The best results are obtained with acid alumina and developing with chloroform–methanol (80:20) for 45–50 min. When using thinner layers (6 g per 15 ml) the solvent must be modified to a chloroform–methanol ratio of 85:15 to obtain the R_F values as listed.

Quite different colors are exhibited by the four curares on spraying the plates or papers with the iodoplatinate reagent. As illustrated in Table I some hydrolytic products are usually detected.

For forensic purposes a double chromatographic identification is usually performed by eluting the spots from the paper chromatogram and by rechromatographing on thin layers.

Qualitative and quantitative determination of *d*-tubocurarine may be obtained by recording the U.V. spectrum of eluted spots (max 280–281 mμ; average value for $E^{1\%}_{1\,cm}$ $^{280.5\ m\mu}$ is 101.6, KLEIN AND GORDON[6]).

TABLE I

PAPER CHROMATOGRAPHY OF CURARE, SOME CURARE-LIKE DRUGS AND RELATED SUBSTANCES
Paper: Whatman No. 1; solvent: *n*-butanol–acetic acid–water (4:1:5); detection: U.V. light and iodoplatinate.

	Compound	R_F	*Colour with iodoplatinate*
1	(Partially hydrolyzed salt of 5)	0.45	pale violet
2	Acetylcholine chloride	0.45	pale violet
3	Decamethonium iodide	0.42	red-violet
4	Choline chloride	0.37	grey
5	Gallamine iodide	0.30	grey-blue
6	*d*-Tubocurarine chloride	0.22	grey-violet
7	Succinylcholine chloride	0.12	violet
8	(Partially hydrolyzed salt of 5)	0.12	pale violet
9	(Partially hydrolyzed salt of 5)	0.02	grey-blue

Biological test in mice

Untreated spots are eluted with distilled water from paper or thin-layer chromatograms and used for a biological test. The eluate is evaporated to dryness, dissolved in 0.1 ml of saline and injected into the tail vein of a mouse. The curare activity is demonstrated by the typical "head drop syndrome" and by the loss of the righting reflexes[5]. According to the dose injected the effect may evolve up to death or recede with a complete recovery of the animal.

RESULTS AND DISCUSSION

The method hereby proposed gives quite satisfactory results for the isolation and the identification of *d*-tubocurarine, decamethonium, succinylcholine and gallamine.

The *isolation* of these poisons cannot be realized by the usual techniques based on extraction with immiscible organic solvents such as ether, chloroform, dichloroethylene, etc. According to our experience[2], the recovery of the curares at alkaline pH is negligible by these methods.

In fact synthetic curares pertain to the group of water- and alcohol-soluble drugs. Therefore the method employed by TEWARI AND BHATNAGAR[7] to isolate gallamine from viscera by ether extraction cannot be regarded as reliable.

The same must be said with respect to *d*-tubocurarine which up to the present time has been studied after organic solvent extraction, both for forensic and pharmacological purposes. According to our experience this method cannot be considered satisfactory, from a quantitative point of view, because the recovery does not exceed 10–15 % owing to the low solubility of *d*-tubocurarine chloride in ether and chloroform.

Therefore, if an extraction is performed with organic solvents on deproteinized blood, organs, urine, etc., the major part of the curares remains in the highly impure aqueous layer. On the other hand the ion exchange method allows a complete separation of the curares from the aqueous solutions and a high purification is thus effected which is then completed by the reineckate precipitation and/or by paper and thin-layer chromatography.

The elution from the cation exchange resin (by means of 2 *N* HCl for the synthetic curares and alcoholic 2 *N* NH_3 for *d*-tubocurarine) leads to the recovery of *ca.* 85–90 % of the curare as may be demonstrated by the spectrophotometric determination of the drug as reineckate. Higher losses are registered when the reineckates are decomposed and the halide salts of the bases are again obtained: it was assumed that, at this stage, the recovery is in the order of 50–55 %, as was also observed by ZAIMIS[8]. However this is of little practical importance in our method because the quantitative determination as reineckates is performed before the regeneration, which is made for the chromatographic and biological identification.

When all the curares studied are simultaneously adsorbed on the cation exchange resin the elution must be made first with acid (four times 2 *N* HCl) and then with alkali (2 *N* alcoholic ammonia), thus permitting a preliminary separation of the curares investigated into two main fractions, the first containing the synthetic curares, the second only *d*-tubocurarine. Only traces of *d*-tubocurarine are in fact found in the HCl eluate and, likewise, only traces of the synthetic curares are found in the alcoholic ammonia eluate.

The *identification* of the curares is mainly carried out by chromatographic and biological tests, *d*-tubocurarine being also identified by U.V. spectrophotometry.

It must be emphasized that in paper chromatography the R_F values of curares can sometimes vary depending on the anions present in the sample to be analysed, as recently demonstrated by LEDERER *et al.*[3] for gallamine. On the other hand, chromatography on alumina thin layers is not affected and allows an easier identification of the compound. In our method paper chromatography is used as a first step in the chromatographic identification for the purpose of purification.

For the biological test, experiments on living animals are to be preferred to those on isolated organs. Among the laboratory animals the mouse is the best, in spite of its low sensitivity to gallamine and decamethonium. Death is effected by intravenous injection of the following doses: 24–32 μg for *d*-tubocurarine, 36–62 μg for gallamine, 10–26 μg for decamethonium, 10–12 μg for succinylcholine.

SUMMARY

A method for the detection of *d*-tubocurarine, gallamine, decamethonium, and

succinylcholine in biological material is described, based on the purification of extracts from body fluids and organs by ion exchange separation on Amberlite IRC 50 resin. The curares are eluted and precipitated as reineckates, which are then regenerated to allow their identification by means of paper and thin-layer chromatography. Thin-layer chromatography with acid alumina layers and chloroform–methanol (80:20) as solvent gives a better resolution. The spots are revealed by the iodoplatinate reagent. The eluates of untreated spots are examined spectrophotometrically in the ultraviolet to detect *d*-tubocurarine and are finally injected in the tail vein of the mice for a biological test.

The method can be considered of particular value for forensic purposes.

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